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Competitive inhibition of TRPV1 – calmodulin interaction by vanilloids

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ABSTRACT: There is enormous interest toward vanilloid agonists of the pain receptor TRPV1 in analgesic therapy, but the mechanisms of their sensory neuron-blocking effects at high or repeated doses are still a matter of debate. Our results have demonstrated that capsaicin and resiniferatoxin form nanomolar complexes with calmodulin, and competitively inhibit TRPV1–calmodulin interaction. These interactions involve the protein recognition interface of calmodulin, which is responsible for all of the cell-regulatory calmodulin–protein interactions. These results draw attention to a previously unknown vanilloid target, which may contribute to the explanation of the paradoxical pain-modulating behaviour of these important pharmacons.

Keywords: TRPV1, Calmodulin, Vanilloid, capsaicin, resiniferatoxin

INTRODUCTION

Vanilloid compounds (Figure 1 inset), such as capsaicin (CAP) and resiniferatoxin (RTX), have long been used as probes for sensory neuronal mechanisms,[1, 2] and have gained therapeutical applications.[3] CAP, the major pungent principle of hot chili peppers,[4] is administered topically (>5%) for purposes of pain relief.[5] RTX, an irritant component of *Euphorbia resinifera*,[6] also displays a prolonged analgesic effect at high local concentrations (up to 100 μM),[7] and is currently undergoing clinical phase II investigations (NCT00804154). These molecules dose-dependently stimulate and subsequently defunctionalize a subset of primary afferent neurons (C and Aδ). Behind these paradoxically opposite effects, there is a cascade of pharmacological processes, in which transient receptor potential vanilloid 1 (TRPV1) plays a central role.[8] TRPV1 is expressed by the peripheral sensory neurons and is responsible for the

pain sensation caused by various physical and chemical stimuli, such as noxious heat, an elevated proton concentration, and endogenous and natural ligands, including vanilloid compounds.[9, 10] Upon stimulation, the receptor opens and Ca^{2+} ions enter the cells causing membrane depolarization and an action potential. The increased Ca^{2+} influx is an important orchestrator of the subsequent neuronal responses: sensitization, desensitization, long-term nerve inactivation, and neurotoxic effects at higher doses.[11] The operation of TRPV1, however, is tightly regulated by the cytosolic Ca^{2+} level,[11] and the neurons possess complex machinery with which to protect themselves against excessive Ca^{2+} influx.[12] In contrast, the existence of these mechanisms cannot prevent the vanilloid-induced impairment of the neuronal functions, and this paradoxical behaviour is the principle behind the analgesic applications of vanilloids. We hypothesized that, besides opening the TRPV1 channel, vanilloid ligands can directly perturb the cellular control over the Ca^{2+}-induced signaling by interacting with a target responsible for the Ca^{2+} sensing and TRPV1 regulation. A number of routes have been suggested for the modulation of TRPV1, in which CaM, a universal Ca^{2+} sensor within the cells (Figure S1),[13] plays a crucial mediating role and can be considered as a hub protein that affects multiple cellular pathways. Phosphorylation on the cytosolic domain of TRPV1 by Ca^{2+}/CaM-dependent kinase II[14] sensitizes the channel, while dephosphorylation of the vanilloid receptor by Ca^{2+}/CaM-modulated calcineurin phosphatase causes desensitization.[15] Further, CaM can play an important role[16] in the desensitization of the receptor as its binding site on TRPV1 overlaps with the ATP-binding site[17] and the phosphorylation and the sensitization[14] of the molecule are therefore impaired. TRPV1 binds CaM at both the N-terminal ankyrin repeats[18] and the C-terminal cytoplasmic domains.[16] It has been postulated that CaM can bridge the cytosolic domains of TRPV1,[19] causing a conformational change after channel opening. The affinity of

TRPV1 for capsaicin has been shown to be dependent on the ability of the channel to bind CaM.[20] There are mechanisms for the vanilloid-induced neuronal dysfunction that are independent of direct channel regulation: disrupted axonal transport[21] and downregulated TRPV1 expression,[22] where Ca^{2+} homeostasis and regulation also appear pivotal.[23] The above literature findings led us to test the direct vanilloid–CaM interactions at the molecular level, which could interfere with Ca^{2+} regulation, and hence with the TRPV1-mediated function of the sensory neurons.

We show here that both CAP and RTX are nM ligands of CaM. A common high-affinity binding site was located for the vanilloids within the hydrophobic core of the Ca^{2+}-stabilized EF-hand motifs, which is responsible for the generic protein recognition properties of CaM. Consequently, we observed the inhibitory effect of CAP and RTX on the TRPV1-CaM interaction.

MATERIALS AND METHODS

CaM expression and purification. Human CaM was cloned into the pET28a(+) vector by standard cloning procedures, and the His6-tagged protein was expressed and purified from an Escherichia coli BL21(λDE3) culture (Novagen). HisPurTM Ni-NTA Superflow Agarose (Thermo Scientific) or TALON® Metal Affinity Resin (Clontech) was used for affinity purification of the His6–CaM construct. The His-tag was removed by thrombin (Sigma-Aldrich) treatment and size exclusion chromatography (Superdex 75) was then used for the final purification step (ÄKTA FPLC). The protein was concentrated by using an Amicon Ultra

(Millipore) centrifugal device, lyophilized in 20 mM HEPES pH 7.0 and stored at -80 °C. Uniformly ^{13}C- and ^{15}N-labelled CaM was purchased from Creative Biolabs.

Design of NMR experiments and signal assignment. The interactions of CaM with the vanilloid ligands CAP and RTX were investigated experimentally by using solution-phase biophysical methods, NMR spectroscopy and isothermal titration calorimetry (ITC). The rather low solubility of these natural pharmacons imposes certain technical limitations. In order to facilitate the solution-phase measurements, a literature solubilisation method was applied, including dissolution in methanol, dilution with water and lyophilisation.[24] As indicated by the NMR measurements carried out in the presence of an internal standard, the final solubilities in aqueous buffer at pH 7.0 and at 30 °C were 320 μM and 18 μM for CAP and RTX, respectively (Figure S2). The solubilized vanilloid ligands could be dissolved incrementally with the protein-containing sample, and the concentration increase was feasible in a stepwise manner. At a CaM concentration of 60 μM, ^{15}N-HSQC NMR titrations were possible whereby the protein could be saturated with the ligands, which appeared sufficient for the detection of any biologically relevant CaM–ligand interaction.

^{15}N-HSQC titrations for $^{15}N/^{13}C$-labelled CaM. ^{15}N-HSQC titration experiments were performed with [$^{15}N/^{13}C$]-CaM (60 μM). Reference 2D heteronuclear ^{15}N-HSQC spectra were acquired for the ligand-free Ca^{2+}-bound CaM. Since a sufficiently concentrated stock solution could not be prepared from CAP and RTX, ligands were added in a solid form (aliquoted and lyophilized from dilute solutions) to the protein sample. Six-nine separate titration points were obtained at different ligand concentrations. At the final titration point, the solution was saturated for the compound. For all the titration points, ^{15}N-HSQC spectra were acquired with 256 increments and the number of scans was 4. The line-broadening analysis was carried out by

using the ^{15}N-HSQC spectra recorded at various ligand concentrations. The line-broadening values were calculated by using the formula $1 - I/I_0$,[25] where I is the signal intensity, and I_0 is the intensity measured in the ligand-free state.

Isothermal titration calorimetry. ITC experiments were carried out with a MicroCal VP-ITC microcalorimeter. Binding experiments for vanilloids were performed in 20 mM pH 7.0 HEPES buffer containing 30 mM $CaCl_2$ at 35 °C. Because of their poor solubility, two types of titration were applied. First, the ligands were in the 1420-µl cell at a concentration of 25 µM and 15 µM, for CAP and RTX, respectively. In individual titrations, 15 µl of CaM solution was injected into the ligand solution from the computer-controlled microsyringe at intervals of 300 s. The concentration of CaM in the titration syringe was 146 or 237 µM. For CAP, CaM solution was placed in the cell at a concentration of 11 µM. In individual titrations, 15 µl of CAP solution was injected. The concentration of CAP in the titration syringe was 300 µM. Control experiments were performed by injecting CaM or CAP into the cell containing buffer with no interacting partner. Experimental data were fitted to the two independent binding sites model (adjustable parameters: n_1, n_2, K_{D1}, K_{D2}, ΔH_1 and ΔH_2) by using a non-linear least-squares procedure. The errors of fitting were estimated with jack-knife resampling.

RESULTS AND DISCUSSION

Ca^{2+}-dependent CaM–vanilloid binding detected by ^{15}N-HSQC titrations. After optimizing the NMR experiments with respect to the low solubility of the ligands (see supporting text), the ^{15}N-HSQC NMR titrations on CaM–Ca^{2+} samples resulted in marked changes in the spectra for both CAP and RTX (Figure 1). When the ^{15}N-HSQC NMR titrations were repeated with Ca^{2+}-

free CaM (20 mM EGTA), no interaction was detected (Figure S3), indicating that the CaM–vanilloid binding is Ca^{2+}-dependent. For CAP, the chemical shift perturbations (CSPs) revealed a complex pattern (Figure 2a-c). Saturation of the CSPs was reached above 3.0 equivalents (Figure S6a,c), and the peaks could be classified into four categories according to their CSP behaviour: (i) peaks exhibiting no CSP; (ii) curved trajectory CSPs with partial ligand-dependent broadening (Figure 2a); (iii) residues undergoing slow/intermediate-exchange that displayed marked ligand-dependent broadening (Figure 2b); and (iv) linear trajectory CSPs in fast-exchange (Figure 2c). These observations supported the occurrence of the CaM–CAP interaction, and suggested that CAP has multiple binding sites on CaM. The slow/intermediate-exchange CSPs pointed to a high affinity ($K_d < 1$ μM) site for CAP, and/or to a slow ligand-induced conformational rearrangement of the protein. The fast-exchange CSPs revealed a weaker ($K_d > 1$ μM) interaction. The turns of the CSPs appeared at around 1.0 equivalent, which indicated a ratio of 1:1 for strong binding, which was accompanied by a weak interaction at multiple sites. For RTX, the CSP saturation was reached at around 2.0 equivalents (Figures 2d,e and S6b,d), and there was no curved trajectory. Line broadening was a general feature for the residues, but some of the peaks did not gain measurable intensity after CSP saturation. These findings indicated that RTX has only one $K_d < 1$ μM binding mode.

High-affinity and inhibitory binding of vanilloids to CaM detected by ITC. CSP data are not well suited for the quantitative estimation of affinity and stoichiometry.[26] Independent quantitative measurements of the thermodynamic properties of the interactions were carried out by means of ITC. For CAP, the ITC conditions could be optimized for both protein in the cell and ligand in the cell experimental set-ups. The protein titration with 11 μM CaM (Figures 3a

and S7) resulted in a two-stage enthalpogram supporting the presence of two separate binding events. Accordingly, the data were fitted by using a model with two independent binding sites: n_1 = 0.97 ± 0.003, n_2 = 1.97 ± 0.005, K_{D1} = 280 ± 25.5 nM, K_{D2} = 1205 ± 86.8 nM, ΔH_1 = 0.24 ± 0.05 kcal mol^{-1}, ΔH_2 = -0.38 ± 0.03 kcal mol^{-1}. The ligand titration with 25 μM CAP yielded the expected fractional stoichiometry relative to CaM, and a long tail passing one equivalent, because of the competition between the high- and low-affinity binding sites (Figure 3b). The non-linear regression of the two independent binding sites model was performed with variable protein concentration: n_1 = 1.01 ± 0.006, n_2 = 2.2 ± 0.05, K_{D1} = 184 ± 8.6 nM, K_{D2} = 1838 ± 148.3 nM, ΔH_1 = -0.93 ± 0.01 kcal mol^{-1}, ΔH_2 = -0.22 ± 0.01 kcal mol^{-1}. It was safe to conclude that both titration set-ups captured the same binding events characteristic of the CAP–CaM interaction. ΔH_1 displayed a difference of 1.17 kcal mol^{-1}, which is an acceptable buffer- and initial concentration-dependent difference. For RTX, only the ligand titration with 15 μM RTX could be carried out, because of the limited solubility of RTX. The enthalpogram indicated a single binding site with the following properties (Figure 3c): n = 1.02 ± 0.003, K_D = 802 ± 24.2 nM, ΔH = -0.91 ± 0.004 kcal mol^{-1}. With the Ca^{2+}-free CaM, there was no ligand-dependent heat response in ITC for CAP and RTX, confirming that the CaM–vanilloid binding was Ca^{2+}-dependent. Further, the ligand binding did not affect the affinity of CaM toward Ca^{2+} (Figure S8).

Having the nanomolar vanilloid – CaM bindings detected, we tested whether these compounds are capable of inhibiting direct TRPV1–CaM interaction. The C-terminal CaM-binding segment of TRPV1, 784-798 (TRPV1-CT, GRNWKNFALVPLLRD)[18] was synthesized and purified via standard peptide coupling methods. Our ITC measurements confirmed the literature finding, and the 1:1 complex of TRPV1-CT–CaM displayed a K_D of 34.6 ± 2.2 nM (Figure 3d).

Repeating the measurement with CAP or RTX as inhibitors in the cell the apparent K_D of TRPV1-CT shifted to 1804 ± 121.5 nM and 186 ± 10.5 nM for CAP and RTX, respectively (Figure 3d, 3e). The significant decrease in the apparent affinity of TRPV1-CT in presence of vanilloids indicated competitive inhibition. By using the Cheng-Prusoff formula with the K_D results for the vanilloids and TRPV1-CT and an estimated low nanomolar intracellular concentration of the CaM-TRPV1 complex, the IC_{50} values were estimated as 0.69 μM and 1.98 μM for CAP and RTX, respectively. The IC_{50} value of 0.69 μM is below the CAP concentrations normally utilized in therapy and in pharmacological experiments (>= 1 μM). For RTX, the IC_{50} of 1.98 μM, is well above the RTX concentration normally applied in pharmacological experiments, but a magnitude below the therapeutical doses used in analgesic therapy.

NMR-guided computational model for the high-affinity CaM – CAP complex. The 1:1 nM binding stage of CAP to CaM afforded the build-up of certain protein–ligand NOEs in the ^{13}C-filtered-3D-HSQC-NOESY spectra, and the 3D-^{15}N-and ^{13}C- NOESY-HSQC recordings were also successful (60 μM CaM and 300 μM CAP), though with limited spectral resolution and signal-to-noise. Nevertheless, the iterative signal assignment (see details in the Supporting Information) unambiguously revealed a total of 290 NOE interactions. The data suggested inter-domain proximity within CaM including contacts between loop S38-Q41 and helix T117-A128, and between the N-terminal loop and the C-terminal helix. Cross-peaks from the methyl groups of the isopropyl moiety of CAP to the side-chain methyls of M124 and I125 could be assigned. Clear NOE contacts from the aromatic 2-H of the vanilloid moiety to the side-chain methyls of M71, M76 and M51 were observed, which determined the orientation of CAP. Further protein–ligand NOEs were found pointing to apolar and aromatic side-chains, but these interactions could

not be unambiguously assigned. For the iterative NMR guided optimization (see details in the Supporting Information) of the CaM-CAP complex, the following restraints were used: (i) NOE-derived protein distance restraints, (ii) NOE- and CSP-derived (Figure S7 and S8) protein–ligand distance restraints, (iii) secondary structure propensity score-derived (SSP, Figure S6)[27] backbone dihedral restraints, (iv) restraints to keep Ca^{2+} ions in the proximity of the corresponding charged groups, and (v) inter-strand H-bond restraints in the β-sheet segments. The solution-phase conformational pool of CaM–Ca^{2+} thoroughly sampled by the Bax and Vendruscolo groups (PDB code: 2K0E)[28, 29] was utilized to select the initial CaM structure, and CAP was manually docked into the assumed cavity already observable in the selected initial structure. It must be emphasized that the 542 structural restraints in total did not allow a refinement of a high-resolution geometry, but the sparse NMR restraints afforded the generation of a low-to-mid resolution model[30] capturing the main features of the CaM-CAP complex (Figure 4). The calculations yielded compact structures and the expected domain contacts, where the N- and C-terminal lobes of CaM formed the CAP binding site with CAP in an extended conformation. The results suggested that the vanilloid binding sub-site was formed by residues L32, N42, T44, E45, L48, M51, I52, V55, I63, F68, M71, M72, K75 and M76 at the N-terminal, and the isopropyl moiety was surrounded by residues F92, L105, M109, V121, M124, I125, F141 and M144 at the C-terminal.

CONCLUSION

We conclude that the vanilloid compounds studied are nM ligands of CaM and competitive inhibitors of the TRPV1–CaM interaction. The IC_{50} values calculated from the dissociation constants (low μM) indicate that the TRPV1 – CaM complex can be a relevant target for

vanilloids at the concentrations applied in the TRPV1-based analgesic therapy (well above 10 μM). The NMR guided model calculations for the CaM–Ca^{2+}–CAP complex resulted in a compact fold where the binding sites of CaM necessary for protein recognition are occupied, which points to the vanilloid ligands as competitive antagonists of CaM in general. In view of the central role of CaM in Ca^{2+} signalling, these results may contribute to the explanation of the mechanism of vanilloid-induced dose-dependent sensitization, desensitization and neuronal dysfunctions.

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Accession codes. Coordinates for the CAP– CaM complex have been deposited in the Protein Data Bank under accession code 2n27. NMR data have been deposited in BMRB under accession code 25588. Uploaded data files and the NMR-STAR file for the ADIT-NMR deposition can be accessible to editors and reviewers using the deposition restart ID 2014-08-02.deposit.bmrb.wisc.edu.80.61812157 from http://deposit.bmrb.wisc.edu/bmrb-adit/access.html

Appendix A. Supplementary data

Additional supporting information may be found in the online version of this article at the publisher's web site:

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Figure 1. An overlay of the ^{1}H-^{15}N-HSQC spectra of CaM–Ca^{2+} (blue) and CaM–Ca^{2+}–CAP (red) (a), CaM–Ca^{2+} (blue) and CaM–Ca^{2+}–RTX (red) (b). The chemical sctructure of the vanilloid ligands are given in inset.

Figure 2. Representative ^{15}N-HSQC chemical shift perturbations upon titration with CAP (a-c) and RTX (d–e). The spectra were recorded for CAP at ligand equivalents of 0.0 (red), 0.36 (orange), 0.72 (yellow), 1.08 (green), 1.43 (beige), 1.80 (coral), 2.15 (turquoise), 2.5 (blue), 3.2 (pink), and 5.0 (purple). The spectra were recorded for RTX at ligand equivalents of 0.0 (red), 0.34 (orange), 0.70 (yellow), 1.4 (green), 2.08 (beige), and 3.5 (pink).

Figure 3. ITC titrations of 11 μM CaM with CAP (a), 25 μM CAP with CaM (b) and 15 μM RTX with CaM (c), 11 μM CaM with TRPV1-CT (d). Competitive ITC titrations of 11 μM CaM with TRPV1-CT as titrant, and CAP (e) and RTX (f) present in the cell as inhibitors at concentrations of 21 μM and 12.5 μM, respectively. The background-corrected binding isotherms (square) were fitted for the two independent binding sites model (solid lines).

Figure 4. NMR-guided computational model for the high-affinity CaM – CAP complex

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